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Hippurate: the natural history of a mammalian-microbial co-metabolite

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Review

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Hippurate: the natural history of a mammalian-microbial co-metabolite

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ABSTRACT.

Hippurate, the glycine conjugate of benzoic acid, is a normal constituent of the endogenous urinary metabolite profile and has long been associated with the microbial degradation of certain dietary components, hepatic function and toluene exposure, and is also commonly used as a measure of renal clearance. Here we discuss the potential relevance of hippurate excretion with regards to normal endogenous metabolism and trends in excretion relating to gender, age, and the intestinal microbiota. Additionally, the significance of hippurate excretion with regards to disease states including obesity, diabetes, gastrointestinal diseases, impaired renal function, psychological disorders and autism, as well as toxicity and parasitic infection, are considered.

TEXT.

Introduction

Hippurate, the glycine conjugate of benzoic acid, is a normal component of urine with a strong association with diet and the intestinal microbiota. Spectroscopic profiling of urine has found hippurate to be a distinguishing feature of many physiological and pathological conditions. Since it is a ubiquitous compound in nature, its utility in elucidating biochemical mechanisms is partially dependent on co-perturbed metabolites (Table 1). As well as being part of the endogenous urinary metabolite profile, hippurate has other specific uses; it has been identified as a biomarker for high dose exposure to certain toxic compounds such as toluene,[1-2] and is also commonly used as a measure of renal clearance.[3-4] Additionally, the ability of a microorganism to hydrolyze hippurate to benzoic acid and glycine has been used extensively as an aid in bacterial species characterization and identification.[5-12] Here we review the origin, etiology and behavior of hippurate in response to physiological and pathological challenges, and consider the effects of gene-environment interaction on its urinary excretion.

History, chemistry and biosynthesis

Hippurate, also known as hippuric acid, benzoylglycine, (benzoylamino)-acetate, is the glycine conjugate of benzoic acid, and has the chemical formula $C_9H_9NO_3$. Hippurate was first identified in urine by Liebig in 1829,[13] and the glycine conjugation of benzoate is thought to have been the first described metabolism reaction,[14] after a report by Alexander Ure in 1841 demonstrated a relationship between the ingestion of benzoic acid and the excretion of hippurate.[15] The reaction was then later confirmed by Wilhelm Keller in 1842.[16] Liebig assigned the name ‘hippuric’ from the Greek word for horse, hippos, as the acid was first

isolated from horse urine. The metabolite is found in high concentrations in the urine of horses and other herbivores and the decomposition of the alkaline hippurates has been attributed to causing the odor associated with horse urine.

Hippurate has a molecular weight of 179.17266 g/mol, a melting point of 187 – 188 °C and a boiling point of 240 °C. In a ^{1}H NMR spectrum (phosphate buffer, pH 7.4; prepared in 8:2 H_2O:D_2O), hippurate is identified by the presence of peaks at 3.97 (doublet, CH_2), 7.56 (triplet, m-CH), 7.64 (triplet, p-CH) and 7.84 (doublet, αCH) ppm, and in ^{13}C NMR spectra by the presence of chemical shifts at (ppm): 179.4, 173.1, 134.8, 131.4 and 129.8.[17-19] In mass spectrometry hippurate has a mass-to-charge ratio of 180.0660 in positive ion analysis, and 178.0504 in negative ion analysis. Fragmentation of hippurate using mass spectrometry predominantly involves loss of glycine.

Urinary hippurate concentrations can be measured using a variety of techniques including ^{1}H [20] and ^{13}C NMR spectroscopy,[21] capillary zone electrophoresis (CZE) and capillary electrochromatography (CEC) coupled with NMR spectroscopy,[22] high performance liquid chromatography (HPLC),[23-24] HPLC coupled with mass spectrometry (HPLC-MS),[25] gas chromatography (GC),[2, 26] GC-MS,[27] solid phase extraction (SPE),[28] colorimetric reaction,[29] immunochromatographic analysis[30] and microfluidic chip-based electrochemical immunoassay.[31]

Some dictionaries and other literature state that hippurate is a substance that is found in the urine of herbivores and rarely in man (‘hippuric acid’ Encyclopaedia Britannica Online, 2011).

Despite this common assertion, more recent measurements of the comparative urinary concentration of hippurate in horse and man in the literature are scarce. However, in a book published in 1885, Gresswell and Gresswell state that hippurate "occurs in very small proportion in the urine of man (less than 1 per cent.) and carnivora, but is present in abundance as alkaline hippurates in that of herbivorous animals.", and reference Ernst von Bibra as finding hippurate to be "from five to fifteen parts in 1000 of the urine of the horse".[32] While these comments might suggest that hippurate is a trace component in urine, when compared to other common organic molecules present in the urine, as detected by ^{1}H NMR spectroscopy for example, it is seen to be one of the dominant signals in the aromatic region of samples obtained from a wide range of species, including man.

The typical urinary concentration of hippurate in man has been measured as 1.83 ± 1.24 mM (absolute), and 2.28 ± 1.43 mM (normalized to creatinine), in healthy subjects,[33] and The Human Metabolome Database reports urinary concentrations of hippurate, relative to creatinine, ranging from 27.92 to 932.66 μmol/mmol creatinine.[34] More recently, the mean 24-hr urinary excretion for hippurate was measured by UPLC-MS/MS as 6284.6 (4008.1) μmol/24-hr in men and 4793.0 (3293.3) μmol/24-hr in women (standard deviation shown in brackets).[35] The findings of this latter study may be more informative than single time point concentration values, as 24-hr urinary excretion measurements account for intra-individual differences and factors such as diurnal variation and diet, which may affect the urinary concentration of both hippurate and creatinine.

The biosynthesis of hippurate occurs within the mitochondrial matrix and requires two reactions,[36] as illustrated in Figure 1. The first reaction involves the conversion of benzoic acid

to benzoyl adenylate via pyrophosphate (PP_i) exchange with ATP. Benzoyl-CoA synthetase catalyses this step and substitutes the adenylate moiety for coenzyme A (CoA). In the second reaction, glycine freely crosses the inner mitochondrial membrane to react with benzoyl-CoA,[37] catalyzed by benzoyl CoA: glycine N-acyltransferase, to produce hippurate.[38-43] Benzoyl CoA: glycine N-acyltransferase has been found to have a higher specific activity compared to benzoyl-CoA synthetase; thus, administration of substances which are able to form CoA esters with the latter enzyme may inhibit the first step in glycine conjugation of benzoic acid, an effect studied using valproate.[44] Evidence suggests that hepatic uptake of hippuric acid is mediated by MCT2, a monocarboxylate transporter, and that there is competition for this transporter with benzoate and L-lactate.[45] Investigators have determined that active renal tubular secretion is the principle elimination route for hippurate,[46] and disruption of this mechanism results in accumulation of hippurate in the blood.[47] Upon ingestion, benzoic acid is biotransformed to hippurate and excreted in urine within 4 hours in man.[48-49]

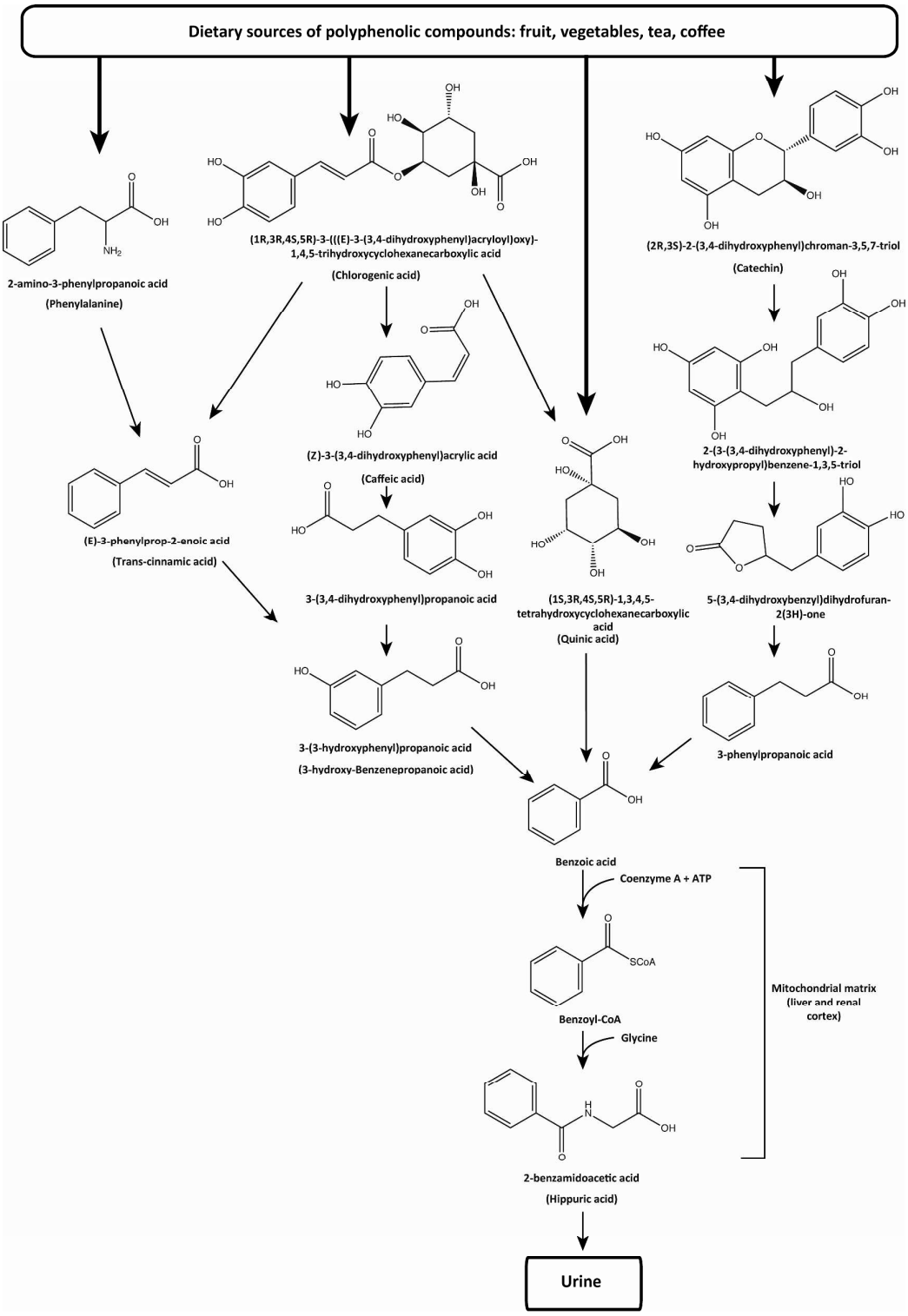

Figure 1. Possible routes of metabolism of dietary polyphenolic compounds, leading to the excretion of hippurate. Adapted from Ure 1841; Kao et al., 1978; Akira et al., 1994; and Clifford et al., 2000.[15,48,63,82]

Rate-limiting factors for hippurate formation

Several factors have been implicated as rate-limiting steps in benzoic acid glycine conjugation, and it has been demonstrated that, following intravenous administration of sodium benzoate, both glycine and CoA supply are limiting factors for hippurate production.[50-51]

Many investigators have concluded that glycine availability is one of the most significant factors in determining the rate of hippurate production. An investigation in rats in 1941 demonstrated that the growth inhibiting-effect of orally administered sodium benzoate was normalized by glycine supplementation.[52] This was further supported by a study in which the co-administration of glycine with sodium benzoate resulted in a normalization of liver serine and glycine concentrations, and also reduced benzoyl CoA accumulation, compared to controls.[53] Studies have also found that glycine administration results in increased hippurate production in man.[54-55]

In addition to glycine, the depletion of CoA has been implicated as a rate-limiting factor. If available glycine supplies become reduced, CoA is trapped as benzoyl-CoA; the result is that free CoA is unavailable to be recycled for the first reaction in glycine conjugation, and thus production of further benzoyl-CoA is halted, limiting the rate of the reaction and the production of hippurate.[50] Further to this, it has also been suggested that the glycine cleavage system may be important in determining the rate of glycine conjugation, due to its role in endogenous glycine catabolism.[51] In addition to rate-limiting factors, evidence suggests that the rate of

glycine conjugation is subject to inter-individual variability in humans, and is normally distributed within the population.[56]

Species differences in glycine conjugation of benzoic acid

Inter-species variation in benzoic acid metabolism has been investigated in several studies[57-61]; as with man, it has been found that 95-100% of benzoic acid is excreted as hippurate in several animals including rodents, the rabbit, cat and the Capuchin and Rhesus monkey. However, differences in the excretion of benzoic acid metabolites were found in animals including the dog, ferret, hedgehog, pig and sheep, with up to 20% of administered benzoic acid excreted as benzoyl glucuronide and benzoic acid. These differences have been attributed to the dose of benzoic acid administered, and also inter-species variation in the glycine and the glucuronic acid conjugation capacity of kidney and liver cells, and the rate of glycine mobilisation.[62-63] In man, hippurate

synthesis occurs in both the liver and cortical cells of the kidney[55, 64-65]; although, it is thought that, due to the anatomical position and larger mass of the liver, this organ is more quantitatively significant for the glycine conjugation of benzoic acid.[50, 56] However, it is also important to note the major site of biosynthesis of hippurate is not always the liver. Indeed, in the dog it would seem that the liver has no part in hippurate synthesis and that this occurs in the kidney.[66]

Gender

Differences in hippurate excretion in males and females have been illustrated in animal studies; Williams et al. found that the urinary metabolite profiles of male and female 8-week-old Zucker rats could be differentiated using PLS-DA modeling of ^{1}H NMR spectral data, with hippurate elevated in the urine of female animals.[67] Higher excretion of hippurate by females was also found by Gavaghan McKee et al. across three different strains of mice (Alpk:ApfCD, C57BL10J and the "Nude mouse").[68]

Gender-associated variation in the excretion of hippurate has also been observed in studies in man. For example, females were found to excrete a significantly greater amount of hippurate compared to males in a study of a Brazilian population ($P<0.05$).[69] A ^{1}H NMR-based study which recruited subjects from Xiamen, China, employed PCA to highlight a trend of increased hippurate excretion in females, although the difference was not statistically significant according to a further ANOVA analysis.[70] Similarly, Psihogios et al. used ^{1}H NMR coupled with PLS-DA, and found that hippurate excretion was higher in the female subjects, but that the difference between gender groups was not statistically significant as judged by an unpaired t-test.[71] As demonstrated by these studies, high inter-individual variation can confound the interpretation of data relating to gender-associated differences in hippurate excretion. In contrast to these studies, Wijeyesekera et al., found that 24-hr hippurate excretion was significantly higher in men compared to women.[35] This difference in findings most likely reflects the intra-individual variation that is accounted for with 24-hr measurements, compared to single timepoint urine collections.

Age

Animal studies have demonstrated a trend of increasing hippurate excretion during the early stages of life. ^{1}H NMR spectroscopic analysis of urine samples from male Wistar-derived rats collected from 4 to 20 weeks of age demonstrated that hippurate excretion was relatively low and highly variable at 4 weeks of age. Excretion then increased as the animals aged, before stabilizing at approximately 8 weeks (Figure 2). It was thought that this pattern was due to the maturation of the gut microbiota and adaptation to an adult diet.[25] These results are further supported by an analysis in Sprague-Dawley rats,[72] and also a life-long study in dogs, in which excretion of hippurate was found to be increased in dogs aged 1.5 years, compared to samples taken from the same animals at 13 weeks old.[73]

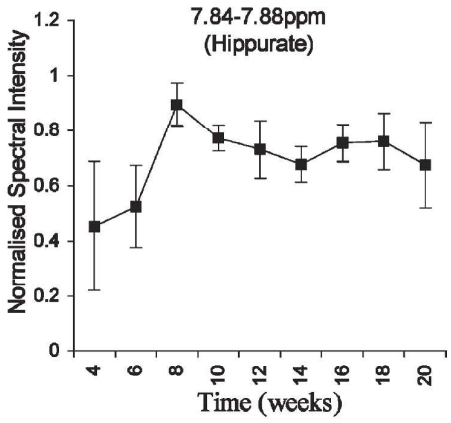

Figure 2. Normalized spectral intensity of hippurate from ^{1}H NMR spectra of urine samples collected from male rats aged between 4 and 20 weeks (Data expressed as mean ± standard deviation). Taken from Williams et al.[25]

Studies in man have also highlighted age-related differences in hippurate excretion. A study which recruited subjects from Minas Gerais, Brazil, with no occupational exposure to toluene, found that subjects aged 36-60 years old (n = 45) excreted significantly more hippurate compared to subjects aged 18-35 years old (n = 70) ($P<0.05$).[69] Wijeyesekera et al also found a trend of increased 24-hr hippurate excretion with increasing age, with higher excretion in individuals aged 50-59, compared to individuals aged 40-49 years.[35] However, in contrast to these findings, a recent study of a Greek population concluded that subjects over 50 years of age tended to excrete lower concentrations of hippurate compared to subjects under 35 years of age.[71] It is difficult to interpret data regarding age-related differences in hippurate excretion in man, due to the high inter-individual and intra-individual variance in synthesis of hippurate.[56, 74] Additionally, several environmental factors have been shown to significantly influence hippurate excretion, such as diet, disease and exposure to certain toxins, which are often difficult to control within the context of a human study, and which may co-vary with age, for example body weight.

Diet

Benzoic acid, and thus hippurate, can be produced from the metabolism of many dietary components including phenylalanine,[75] quinic acid,[76] shikimic acid,[76-78] and various phenolic compounds such as chlorogenic acid and (+)-catechin (see Figure 1).[79] Hippurate excretion has long been associated with the metabolism of polyphenol-rich components of the diet such as vegetables, fruit, tea and coffee.[80-82] The large polyphenolic molecules found in the diet, such as chlorogenic acid and (+)-catechin, are transformed via microbial and mammalian co-metabolism through a series of steps, resulting in a range of simpler aromatics, such as

phenylpropionic acid, which can then be further metabolized to produce benzoic acids.[79, 82-83] Also, benzoic acid used as a food preservative is a further source of hippurate synthesis.

There has been much interest in the key polyphenolic molecules found in the human diet due to their antioxidant potential and associated impact on health. As such, various studies have investigated the metabolism and absorption of polyphenols such as chlorogenic acid,[84] quinic acid[76] and cinnamic acid,[85-87] and in each case, hippurate has been identified as a predominant urinary metabolite.

Additionally, a number of studies have shown that increased hippurate excretion can result from the ingestion of specific dietary components, including chamomile tea,[88] Gingko biloba,[89-90] apple cider,[91] sweet potato,[92] cranberry,[93] and other edible fruits.[94] The metabolism of tea and the bioavailability of tea flavanoids has been the subject of particular interest,[79, 82, 95-98] and it has been shown that hippurate is the main urinary metabolite from consumption of black[82, 95] and green[96] tea, with a three-fold increase in hippurate excretion reported following the consumption of eight cups of black tea per day.[82]

It is thought that hippurate production, following consumption of tea, is due to the metabolism of catechins and related polymers such as theaflavins and thearubigins.[99] The phenolic molecules originating from tea are poorly absorbed in the small intestine, and are thus available for metabolism by the colonic microbiota.[100] The microbial species cleave the catechin ring into valerolactones, which are then metabolized to phenylpropionic acids. These molecules are absorbed and metabolized in the liver via β-oxidation to produce benzoic acid, before glycine

conjugation and excretion as hippurate.[83, 101] Olthof et al. demonstrated the significance of the gut microbiota in metabolizing polyphenolic compounds when it was shown that, following administration of cholorogenic acid and tea phenols, subjects with an intact colon metabolized roughly half of the phenols to hippurate; whereas in subjects without a colon, only trace amounts of phenolic acid metabolites were excreted.[79]

There has also been interest in how black and green tea may differ in their effects on human metabolism,[97] and much debate as to the superiority of green tea with respect to conferring health benefits. Van Dorsten et al. compared the effects of black and green tea on metabolism in 17 male subjects, in a randomized cross-over study. Urine samples were analyzed using ^{1}H NMR spectroscopy coupled with PCA and PLS-DA. Consumption of both black and green tea resulted in increased excretion of hippurate and 1,3-dihydroxyphenyl-2-O-sulfate; however, PCA demonstrated separate clustering of samples for black and green tea, with green tea consumption resulting in the excretion of several unidentified aromatic metabolites. Additionally, subtle differences in the effect on endogenous metabolism were found between green and black tea, with green tea causing an increase in the excretion of TCA-cycle intermediates, such as pyruvate, oxaloacetate, 2-oxoglutarate, succinate and citrate.

Certain studies have examined the impact of entire food groups and diets on urinary metabolite profiles. In a recent ^{1}H NMR spectroscopy-based metabonomic study, urine samples were collected from 41 male and 40 female omnivorous (OMN) subjects, aged between 23 and 55; and 42 male and 38 female lacto-vegetarian (VEG) subjects, aged between 18 and 40. VEG participants were observed to excrete higher amounts of hippurate than those on a OMN diet. The greater phenolic content of the lacto-vegetarian diet was implicated in such differences. In

addition, succinate, citrate and formate excretion was also found to be higher in VEG samples, whereas TMAO, taurine, glycine, phenylalanine and methylhistidine were higher in OMN samples. However, significant differences in the lifestyles of VEG and OMN subjects may also have contributed to the differences in urinary metabolites observed.[70]

Similar findings were described by Walsh et al. in a study which compared urinary metabolite profiles from subjects on a low-phytochemical diet (LPD) in which fruit and vegetables were absent, a standard-phytochemical diet (SPD) which did contain sources of fruits and vegetables, and also a 'normal diet' (ND), which allowed subjects to follow their usual dietary routine. Samples were analyzed using ^{1}H NMR spectroscopy and mass spectrometry, and PCA and PLS-DA revealed that the LPD samples contained lower amounts of hippurate compared to the SPD and ND, further supporting the association between phytochemical intake and hippurate excretion.[102] Further to this, a recent study by Fardet et al. highlighted the potential impact of whole grains on human metabolism; it was found that a change in diet from 60% refined wheat flour to 60% whole-grain wheat flour resulted in an increase in the excretion of hippurate, a result the authors attributed to the increased polyphenol content of the latter diet [103]. In addition, specific dietary interventions have also been shown to result in reduced hippurate excretion; in a recent NMR-based metabonomic study it was found that a milk protein diet reduced the urinary excretion of hippurate. The authors attributed this result to changes in gut microbial metabolism.[104]

The influence of a diet high in benzoic acid has also been demonstrated; Zuppi et al. investigated the difference in urinary metabolites of subjects from different geographical locations, and thus differing diets. A comparison of urine samples from 25 subjects living in

Rome, consuming a typical Mediterranean diet, and 25 subjects living in Ny-Alesund (Svaldbard, Norway), consuming a diet high in preserved food, revealed that the latter group excreted higher amounts of hippurate. This difference was thought to have derived from the high benzoic acid content of preserved food.[105]

Microbiota

Research is ongoing into the wider importance of the intestinal microbiota in host health and disease[106-108]; a number of studies have demonstrated the importance of the gut microbiota in contributing to the excretion of a range of metabolites including TMAO, indoxyl sulfate, trimethylamine, phenylacetylglutamine, and hippurate, and as such they are often referred to as urinary mammalian-microbial co-metabolites. The significance of perturbation in hippurate levels is often attributed to gut microbial activity; certainly, germ free animals do not excrete hippurate, and on exposure to the environment, at around 2 weeks post exposure, hippurate becomes the dominant aromatic metabolite (Figure 3).[109-112] Likewise, treatment of rats and mice with antibiotics have been shown to eliminate hippurate (Figure 4), and as with the germ free rats, concentrations have been shown to stabilize and reach a maximum at around three weeks post dose.[113-114]

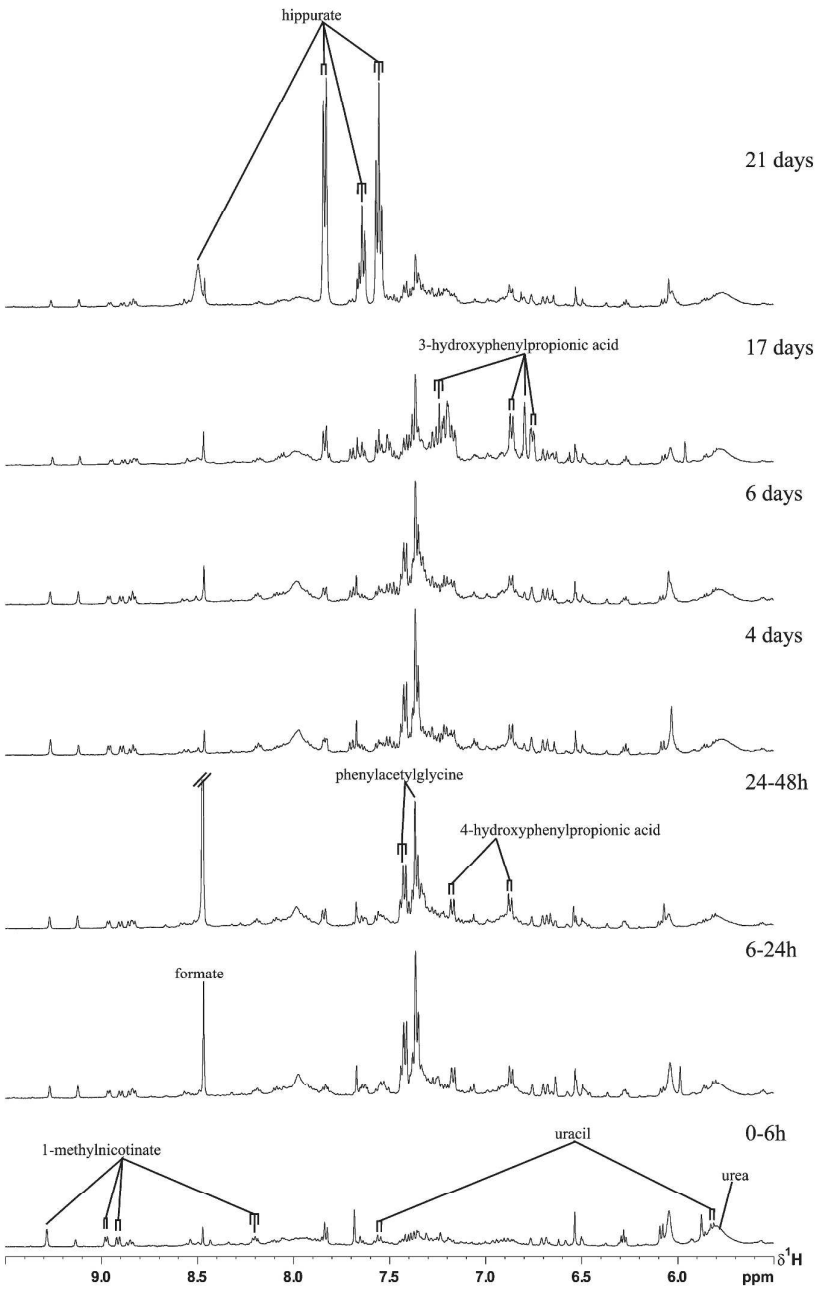

Figure 3. Expansion of the aromatic region (δ9.5-5.5) of a 500 MHz ^{1}H NMR spectrum of rat urine at selected time points (shown on the right). Time 0h represents the introduction of the germ free rats to a standard laboratory environment. Note that by day 21, hippurate is a dominant species in the aromatic region of the spectrum. Taken from Nicholls et al.[112]

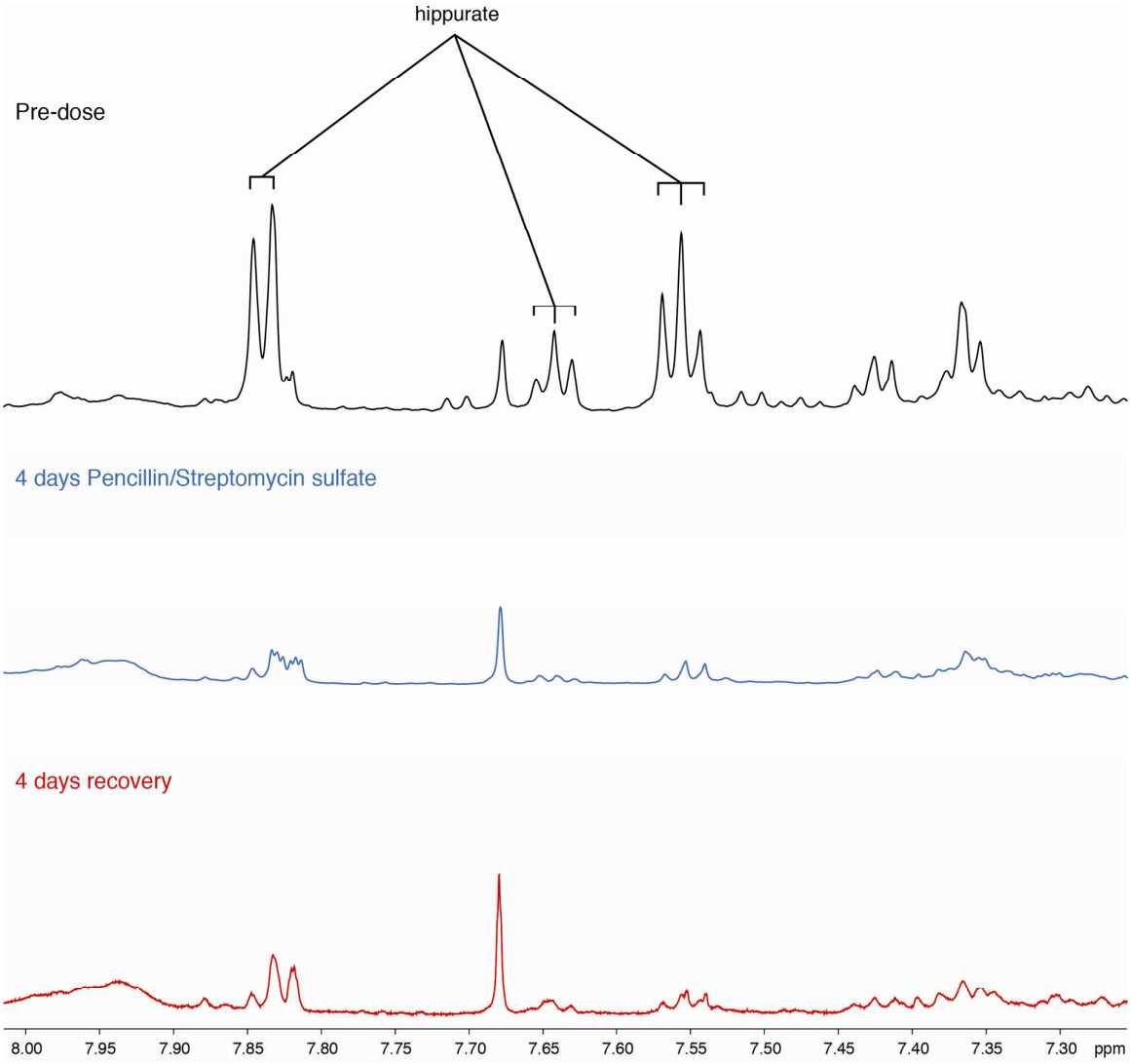

Figure 4. Aromatic region of 600 MHz ^{1}H NMR spectra of urine obtained from a rat pre-dose, 4 days treatment with penicillin and streptomycin sulfate and 4 days after the final dosing. Adapted from Swann et al. [114]

The role of the gut microbiota in the metabolism of polyphenolic compounds has been investigated extensively via the use of orally administered antibiotics in 'conventional' animals possessing an established intestinal microbiota. Results have shown that antibiotic-induced suppression of the gut microbiota results in a reduction in the excretion of hippurate and related

metabolites (Figure 4).[113, 115-119] In addition, it has been shown that 'germ free' animals, born into and maintained in a sterilized environment, and lacking in conventional intestinal microbiota, are unable to excrete hippurate or related metabolites, thus confirming their microbial origin.[109-111] Furthermore, it was shown that when germ free animals were introduced to a standard non-sterile laboratory environment[112] or inoculated with intestinal bacteria,[120] excretion of hippurate and other phenolic acids significantly increased. Taken together, these results indicate that the presence of an established gut microbiota is fundamental to the metabolism of phenolic dietary components, and consequently the production of hippurate.

There is evidence to suggest that high-molecular-weight polyphenolic compounds are poorly absorbed in the small intestine[121-122]; for example, it was calculated that only 33% of administered chlorogenic acid was absorbed in the small intestine of ileostomy subjects.[100] This allows for a large proportion of consumed polyphenolic compounds to reach the colon where the majority of commensal bacteria reside (10^9-10^{12} cfu/ml). In vitro and in vivo studies have demonstrated the capacity of the intestinal bacteria in metabolizing polyphenolic compounds through a variety of reactions, including dehydroxylation, reduction, hydrolysis, decarboxylation and demethylation.[109, 123-127] For example, hydrolysis of chlorogenic acid by the gut microbiota yields caffeic acid and quinic acid; the microbiota are then able to reduce the former to 3,4-dihydroxyphenylpropionic acid, and then dehydroxylate this to 3-hydroxyphenylpropionic acid (3-HPPA). Both 3-HPPA and quinic acid can then be further metabolized to benzoic acid, and excreted as hippurate.[84, 101, 125-126]

Certain bacterial species have been linked to specific reactions involved in the metabolism of phenolic compounds.[128] Accordingly, it has been postulated that changes in the species

population or activities of the intestinal microbiota may result in a difference in the metabolic processing of polyphenolic compounds within the colon and, consequently, a difference in the yield of microbially derived urinary metabolites. Thus, the urinary metabolite profile can be an informative tool in the analysis of the intestinal microbial profile.

Further to this, certain studies have speculated that a potential redistribution of the microbiota has been caused by a change in diet[101] or cage environment.[115, 129] For example, in a study of Wistar rats by Phipps et al., dietary modulation was found to cause a change in the aromatic excretion profile, such that excretion of 3-HPPA was replaced by hippurate. Interestingly, when the animals were returned to the original Special Dietary Services (SDS) diet, excretion of hippurate persisted. It was proposed that, in addition to the precursors available in the diet, the absence and presence of urinary hippurate and 3-HPPA was influenced by variation of the intestinal microbiota, and that a change in diet had potentially caused a redistribution of the microbiota, resulting in the production of hippurate as the primary excretion product, regardless of the specific diet.[101] See Figure 1 for the metabolic pathways involved.

Disease states and disorders

Obesity.

There is gathering evidence to suggest that alterations in the functional intestinal microbiome-host relationship are associated with the development of obesity; in addition, hippurate has

been highlighted as a discriminatory metabolite in a number of metabonomic comparisons of the urinary profiles of obese and lean subjects.

The intestinal microbiota are beneficial for the host in several respects, with one key activity being energy extraction and metabolism of otherwise indigestible dietary components. The relationship between the intestinal microbiota and an increase in caloric extraction and promotion of fat deposition, was clearly demonstrated by Bäckhed et al.; conventional mice were found to have 42% more total body fat compared to germ-free mice, despite consuming 29% less food than the germ-free animals. Further to this, the conventionalization of germ-free mice with intestinal bacteria from the cecum of conventional mice produced a 57% increase in total body fat content after 14 days, despite an associated decrease in chow consumption.[130]

Several studies have demonstrated a difference in the species populations of the microbiota of lean and obese individuals, most noticeably in terms of the relative proportions of the two dominant phyla of anaerobic bacteria, the Bacteroidetes and Firmicutes[131-133]; a finding also supported by animal studies.[134-137]

A relatively small human study by Ley et al. explored the relationship between caloric intake and the impact on intestinal microbial ecology; an analysis of the intestinal microbiota revealed that 12 obese individuals had fewer Bacteroidetes ($P<0.001$), and a greater number of Firmicutes ($P=0.002$), compared to lean controls. They were then randomly assigned to either a fat-restricted (FAT-R) or carbohydrate-restricted (CARB-R) low calorie diet. Over the course

of one year, the relative abundance of Bacteroidetes increased, and the number of Firmicutes decreased, and the increase in Bacteroidetes correlated with percentage loss of body weight.[131]

This shift in the relative proportions of Firmicutes and Bacteroidetes during weight loss was also seen in a study of 39 overweight and obese adolescents, using fluorescence in situ hybridization (FISH) analysis.[133] Additionally, a metagenomic study of 154 individuals, comprising adult monozygotic and dizygotic twin pairs concordant for leanness or obesity, and their mothers, found that obesity was associated with phylum-level alterations in gut microbial ecology and reduced bacterial diversity.[132]

However, several studies have indicated that the relationship between obesity and the intestinal microbiota goes beyond the relative proportions of Firmicutes and Bacteroidetes. For example, studies have found higher numbers of Bacteroidetes during excessive weight gain, or in obese compared to lean individuals,[138-139] or no difference in the proportions of Bacteroidetes between obese and lean groups.[140] Thus, it is clear that it is too broad a taxonomic description to compare microbiota in terms of Bacteroidetes,[141] and that there may be complex associations with obesity at the genus level, as has been found with Bifidobacteria.[142] Taken together, these data imply that small changes and very specific modulation of intestinal microbial ecology may be related to the development of obesity.

In addition to the evidence of perturbations in intestinal bacteria associated with the obese phenotype, a number of studies have shown that this condition results in an altered urinary metabolite profile, compared to lean subjects.[143-144] For example, Calvani et al. used a ^{1}H

NMR-based metabonomic approach to analyze the urinary metabolite profiles of 15 morbidly obese individuals and age-matched healthy controls. Significant separation of the obese and lean subjects was achieved using PLS-DA. Hippurate was identified as the most important discriminant metabolite, and was found to be lower in the samples from the obese individuals.[144] Decreased hippurate excretion was also previously seen in an ^{1}H NMR-based metabonomic analysis of Zucker (fa/fa) obese rats, compared to Zucker (fa/-) lean controls.[145] Further to this, life-long caloric restriction has also been linked to variation in hippurate excretion; Wang et al. concluded that life-long diet restriction altered the activities of the gut microbiota, as demonstrated by variation in aromatic metabolites and aliphatic amine compounds, with hippurate excretion consistently higher in calorie-restricted dogs, compared to controls.[73]

Taking into consideration the evidence relating to the characterization of an 'obese microbiome', it is likely that the differences in hippurate excretion seen in these studies are due, at least in part, to functional or compositional differences in intestinal microbiota between the obese and lean individuals. Indeed, a recent study by Waldram et al. combined a ^{1}H NMR-based metabonomic analysis of urinary metabolite profiles with FISH and DGGE analyses of variation in the structural composition of the intestinal microbiome. It was shown that Zucker (fa/fa) obese rats excreted less hippurate compared to Zucker (-/-) and (fa/-) lean controls, and that this correlated with lower Bifidobacteria counts in the obese rats.[146] More recent studies however, suggest that the functional relationship between the host and microbiome may be far more complex than first appreciated. Significant variation in the composition of the gut microbiota has been shown to be subject to environmental effects, such as animal housing (so called "cage-effects"), whilst the urinary hippurate profile may be more dependent on host phenotype, rather than particular populations of microbiota per se (Lees et al, in preparation). It

is hoped that correlation of urinary metabolite profiles, to measure the metabolic output of host-intestinal microbiome interactions, with bacterial compositional profiling approaches, such as next generation sequencing, will give further insight in to the significance of functional differences in host-microbiome interaction in health and disease.

Diabetes.

Urinary metabolite profiles have been used by a number of investigators to investigate differences in metabolism relating to diabetes. Zuppi et al. performed ^{1}H NMR spectroscopic analysis of urine from children and adolescents with type 1 diabetes; the spectra were normalized to the signal of creatinine and metabolites were quantified using peak height. Comparisons with samples from sex and age-matched healthy individuals revealed that hippurate excretion was significantly elevated in diabetic individuals ($P < 0.001$).[147] The authors suggested that the increased hippurate excretion might have been due to increased glomerular filtration rate, a characteristic of type 1 diabetes,[148] or other differences in renal function. It was also proposed that the diabetic individuals had increased availability of hepatic acetyl-CoA, and thus an increased capacity for the glycine conjugation of benzoic acid. However, the authors made no mention of controlling for diet-related differences between the groups, and it is possible that this influenced the concentration of hippurate in the samples.

Increased hippurate excretion has also been seen in studies of type II diabetic patients; investigators have found differences between type II diabetic patients and healthy controls by supervised multivariate statistical analysis of the ^{1}H NMR spectra of urine samples [149] and also

through quantification of metabolites.[150] Doorn et al. found that hippurate excretion was positively correlated with glycosuria and glycohemoglobin, which might reflect an association between hippurate excretion and deterioration of metabolic control, or alteration in renal function in type II diabetic patients. Further to this, the authors used principal component discriminant analysis (PC-DA) to investigate the effect of rosiglitazone, an antidiabetic pharmaceutical, on metabolism in type II diabetics and healthy controls. It was found that treatment resulted in a reduction in hippurate excretion in diabetic patients, and no significant treatment-related changes in metabolite excretion in the healthy controls were found.

In contrast to these findings, Salek et al. used a ^{1}H NMR-based metabonomic approach to compare the urinary metabolite profiles of type II diabetes patients with healthy volunteers, and identified hippurate to be lower in the urine of type II diabetes patients compared to controls, using PLS-DA.[145] However, it was not made clear how significantly signals from hippurate contributed to the model, and thus, how significant the difference in excretion was between the groups.

It is important to note that type II diabetes is often related to obesity and other components of 'the metabolic syndrome', as they are risk factors for the disease.[151] These associated complications may also influence hippurate excretion, and as such, consideration should also be given to differences in diet, and potential differences in microbiota as a result of the 'obese microbiome', when interpreting results.

Blood pressure and atherosclerosis.

A correlation of increased blood pressure with reduced hippurate excretion was established in a study of human populations; the International Study of Macronutrients and Blood Pressure (INTERMAP) used an ^{1}H NMR–based metabonomic approach to investigate the urinary metabolite phenotype variation across and within population samples from China, Japan, the United Kingdom, and the United States. The excretion profiles varied across different geographical populations, and gut microbial-mammalian co-metabolites, including hippurate, were found to be discriminatory. This analysis was then linked to data on the individuals regarding blood pressure, and hippurate was found to be inversely associated with blood pressure in multiple linear regression models. It was postulated that this association might reflect differences in diet and gut microbial activity between the different populations.[152]

An association between hippurate excretion and blood pressure has also been explored using the urinary metabolite profiles of spontaneously hypertensive rats (SHR). Akira et al. found that hippurate excretion was lower in SHR compared to normotensive Wistar Kyoto rats, using ^{1}H NMR spectroscopy combined with PCA.[153] The authors considered that this difference in hippurate excretion might reflect the differing microbiomes of the two strains, due to differences in host genetic and metabolic factors, as the diet was kept the same for both groups. The authors note that the animals were bred under the same circumstances, however, it cannot be ruled out that differing cage environments may also have contributed.

In addition to an association with blood pressure, hippurate has also been identified as a potential biomarker in atherosclerosis rat models. Using ultra fast liquid chromatography

coupled with IT-TOF mass spectrometry (UFLC/MS-IT-TOF) to analyze the urine of diet-induced atherosclerotic rats, Zhang et al. identified several discriminatory metabolites using multivariate statistical analysis, with hippurate excretion found to be higher in the atherosclerotic rats, compared to controls.[154] This result contrasts with other studies that have shown hippurate to be associated with a lean phenotype and with ingestion of a flavanol rich diet.[82, 95-96, 144] No specific mechanism was offered by the authors with regard to this discrepancy in hippurate excretion. It is plausible that higher hippurate excretion may relate to the progression of atherosclerosis however, differing dietary regimes between the two groups as well as potential differences in cage environment may also have contributed to the observed results.

Gastrointestinal Diseases.

There is increasing evidence to suggest that the gut microbiota contribute to the development of various gastrointestinal diseases such as inflammatory bowel disease, colorectal cancer and irritable bowel syndrome.[155-157] Molecular techniques have been used in several studies to demonstrate differences in the species populations of mucosal or fecal bacteria in IBD patients compared with healthy individuals,[158-165] with evidence of a decrease in microbial species diversity in IBD.[166] Further to this, urinary metabolite profiles have been shown to differ between individuals with Crohn's disease and ulcerative colitis and those sampled from healthy controls.[167-168] In an analysis of urine samples using ^{1}H NMR spectroscopy combined with multivariate statistics, Williams et al. found that hippurate was the dominant metabolite for discriminating between Crohn's disease patients and healthy controls, and also between patients with Crohn's disease and ulcerative colitis. The study also compared quantified levels

of hippurate, expressed as a relative index to total spectral integral. Hippurate excretion was significantly lower in patients with ulcerative colitis compared to controls (P=0.0001), and lowest overall for individuals with Crohn's disease. In an attempt to minimize confounding factors, detailed histories of food intake were kept for all participants and analyzed for potential differences in tea intake and other dietary sources of precursors for benzoic acid and hippurate. Statistical analysis of these dietary factors revealed no differences between groups, and thus, the authors postulated that the differences in hippurate excretion were a reflection of differences in the intestinal microbiota of the different disease groups. Following this study, Williams et al. provided further evidence in support of this theory regarding Crohn's disease patients specifically. In an analysis of urinary NMR spectra, baseline urinary hippurate concentrations were found to be significantly lower in patients with Crohn's disease, compared to controls, despite no significant difference in the diets of the two groups. Further to this, in order to investigate whether the reduced concentration of urinary hippurate seen in the Crohn's disease patients was due to an intrinsic deficiency in glycine conjugation, a dose of sodium benzoate was administered to both groups, and the concentration of urinary hippurate measured. The peak excretion of hippurate was seen at one hour post-dose, and no significant difference was found between the control and disease groups. The investigators concluded that the patients with Crohn's disease did therefore not have a deficit in the conjugation of benzoate, providing strong evidence for dysbiosis of the microbiome underlying the reduced hippurate excretion seen in Crohn's disease patients.[169]

Renal function.

Hippurate has been characterized as a protein-bound uremic toxin,[170-171] accumulating in serum when renal clearance is impaired.[172] It has been suggested that the renal clearance of endogenous hippurate is a useful indicator of other alterations in renal secretion that are associated with reduced expression of organic anion transporters in chronic renal failure.[46] In this manner hippurate can be used in addition to urea as a marker for the assessment of the efficacy of dialysis.[173]

The kidneys serve as both a site of glycine conjugation of benzoic acid, and also as the primary elimination route for hippurate via renal tubular secretion. As such, altered renal functioning due to disease may result in the disruption of hippurate production or its elimination. A trend of reduced hippurate excretion has been noted by investigators of certain renal disorders; for example, Bairaktari et al. investigated renal tubular damage in patients with obstructive jaundice and noted reduced hippurate excretion in patients compared to age and sex-matched controls.[174] Further to this, the ^{1}H NMR signal of hippurate was absent from the urine of a patient presenting with rhabdomyolysis, and associated renal tubular malfunction. Following treatment of the condition, and subsequent improvements to renal function, hippurate excretion was observed to increase.[175] Additionally, patients with glomerulonephritis (GN) have also been found to excrete reduced hippurate. Psihogios et al. analyzed the urine samples of patients with mild, moderate and severe GN using an ^{1}H NMR-based metabonomic approach.[176] OPLS-DA models revealed metabolic differences between samples from GN patients and healthy controls, and also differentiation between those with mild and severe lesions, with hippurate identified as contributing significantly to the model. The onset of tubulointerstitial lesions were found to be associated with a decreased excretion of hippurate, while further deterioration resulted in the total depletion of urinary hippurate. The authors proposed that this result was

indicative of a potential alteration in metabolism, or a reflection of the efficacy of tubular secretion, as this is the primary elimination route for hippurate.[46]

More recently, decreased hippurate excretion was observed in patients with primary renal hypouricemia (PRH), a rare condition that results in increased renal clearance of urate. OPLS-DA models of ^{1}H NMR spectral data demonstrated separation between urine samples from PRH patients and sex and age-matched healthy individuals, with hippurate identified as an important metabolite underlying the separation. The decreased excretion of hippurate in the PRH patients was thought to have resulted from compromised tubular secretion, or as a result of reduced availability of glycine, since increased excretion of this amino acid was seen in hypouricemic individuals.[177]

Psychological disorders.

Variation in hippurate excretion has long been investigated in patients with anxiety, depression, schizophrenia and other psychological disorders. Investigators have described decreased excretion of hippurate in patients with schizophrenia and depression,[178-179] and elevated hippurate excretion during episodes of anxiety.[180-182] Diminished hippurate excretion in schizophrenic patients is thought to relate to reduced availability of glycine,[178] however the connection between glycine and this disorder remains to be elucidated.[183]

More recently, an association between anxiety and hippurate excretion has been shown using a metabonomic approach; Martin et al. described a 'specific metabolic signature', characterized by ^{1}H NMR and MS, which was associated with individuals with high anxiety traits. It was found that individuals with high anxiety had differing concentrations of gut microbially derived metabolites, including higher hippurate excretion. Interestingly, daily consumption of dark chocolate for two weeks was found to reduce urinary excretion of cortisol and catecholamines, in subjects with high anxiety, and also partially normalized stress-related differences in hippurate, as compared to controls.[184]

Variation in hippurate excretion was also observed after Sprague-Dawley rats were subjected to acute stress and chronic unpredictable mild stress, in the form of 'cold exposure', 'forced swim', and 'chronic unpredictable mild stress' (CUMS) tests.[185] This was further investigated by Zheng et al. in an UPLC-MS-based metabonomic study of an animal model of depression produced by CUMS, and it was found that CUMS-treated rats were characterized by increased hippurate excretion, compared to controls.[186] Variation in plasma hippurate concentrations in an animal model of depression has also been noted.[187] However, it is unknown to what extent such animal models of stress and depression are able to give insight in to human psychological disorders and any associated alteration in metabolism.

Autism.

Autism spectrum disorders (ASDs) are primarily characterized by a complex range of socio-psychological and neurodevelopmental problems, with great variation in the severity and

specific nature of deficits among individuals.[188] In addition to these clinical signs, autism is associated with a range of metabolic, immunological and gastrointestinal problems.[189-191] In order to investigate the metabolic abnormalities associated with autism and potentially identify diagnostic markers, Yap et al. analyzed the urine samples of 39 autistic children, their unaffected siblings, and age-matched controls, using ^{1}H NMR spectroscopy and multivariate statistical analyses.[192] The relative patterns of mammalian-microbial co-metabolites, including dimethylamine, phenyacetylglutamine and hippurate, were found to differ between the samples from autistic children and controls. The investigators observed a trend towards decreased hippurate excretion in samples from autistic children, although a non-parametric 2-tailed Mann-Whitney test revealed that the difference was not statistically significant. Additionally, a prior study by Lis et al. also found an association between hippurate excretion and autism using high-resolution ion-exchange chromatography. After analysis of urine samples from 19 autistic children, a trend of decreased hippurate excretion was described, compared to controls.[193] Yap et al. suggested that the differences in microbially derived metabolites could be related to gastrointestinal dysfunction associated with autism, and specifically, differences in the clostridial species diversity of the microbiota.[194-195]

Toxicity.

Variation in hippurate excretion has been observed following administration or exposure to a number of compounds associated with toxicity. As with altered renal functioning due to disease, nephrotoxins also have the potential to affect both hippurate synthesis and elimination. Indeed, decreased hippurate excretion has been observed following the administration of a

variety of nephrotoxins including mercury II chloride, and other S3 proximal tubular nephrotoxins; ifosfamide and calcineurin inhibitors.[196-204]

Inhibition of proximal tubular secretion and increased hydrolysis of hippurate by kidney aminoacylase have been cited as possible mechanisms for reduced hippurate excretion in the case of ifosfamide[201-202] and calcineurin inhibitors,[204] respectively. However, certain nephrotoxins, such as gentamicin and cephaloridine, act as antibiotics and therefore, modulation of the microbiota is the likely mechanism for the variation in hippurate excretion observed with these compounds.[196, 198-200, 205] Modification of the microbiota has been associated with a number of other toxic compounds, resulting in either an increase[206-208] or decrease[209] in hippurate excretion.

In addition to nephrotoxins, certain hepatotoxins, including hydrazine, methylene dianiline, galactosamine and Huang-yao-zi, have also been linked with decreased hippurate excretion.[203, 210-212] The liver is thought to be the primary site of glycine conjugation of benzoic acid in many species, and as such, disruption of this reaction may be a possible mechanism for reduced hippurate excretion. Liu et al. proposed that administration of Huang Yao-zi caused damage to hepatic mitochondria, resulting in a shortage of ATP, and thus inhibiting the ATP-dependent process of hippurate synthesis.[211] The hippurate ratio has been highlighted as a potential preoperative tool for the assessment of functional hepatic reserve in cirrhotic patients selected for liver resection.[213]

Variation in hippurate excretion has also been linked to compounds with more generalized, multi-system organ toxicity such as cadmium[214] and selenium.[215] In addition, inhibition of glycine conjugation has been proposed as a possible cause of reduced hippurate excretion following administration of chlorophenoxyacetic acid herbicides[216] and 1,3-butadiene.[217]

Increased hippurate excretion also results directly from the metabolism of certain toxic compounds such as toluene,[1, 218] N-ethylbenzamide[219] and gasoline.[220] Toluene is primarily metabolized via hydroxylation to benzyl alcohol by members of the cytochrome P450 superfamily.[221] Benzyl alcohol is then metabolized to benzaldehyde and benzoic acid, with the majority of benzoic acid glycine conjugated and excreted as hippurate.[222-223] Similarly, benzoic acid is the source of hippurate excretion following exposure to N-ethylbenzamide; it is first metabolized via hydrolysis to ethylamine and benzoic acid, with the majority of the dose excreted as hippurate.[219] Accordingly, an increase in the excretion of hippurate has been observed following environmental exposure to such compounds[1, 224] with hippurate cited as a urinary biomarker for high dose exposure to toluene.[1, 218]

Parasitic infection.

Reduced hippurate excretion has been associated with a number of parasitic infections, investigated using a metabonomic approach; these have included Schistosoma mansoni, Schistosoma japonicum, Trypanosoma brucei brucei, Echinostoma caproni and Necator americanus.[225-231] Moreover, hippurate excretion was found to be negatively correlated with the levels of worm burden.[230] In addition to a reduction in hippurate excretion, certain studies also

found alterations in other mammalian-microbial co-metabolites, suggestive of an alteration in the species population or activities of the microbiota. Additionally, infection with either E. caproni, S. mansoni or S. japonicum resulted in reduced hippurate excretion and increased phenylacetylglycine, p-cresol-glucuronide and trimethylamine excretion suggesting that these trematodes share similarities in how they influence the gut microbiota.[225-227]

It has also been suggested that decreased hippurate excretion following S. mansoni infection in mice may have been in part due to altered glycine conjugation,[225] as S. mansoni infection has been previously associated with increased cytochrome P450 activity,[232] which may have influenced phase II metabolism.

Co-variation with other urinary metabolites

In several of the metabonomic studies discussed in this review, TCA cycle intermediates, in particular citrate, succinate and 2-oxoglutarate, have often been found to co-vary with hippurate excretion in the same direction (i.e. either increased or decreased in biofluids) (Table 1). In certain cases this co-variation can be explained by the link between hippurate formation and mitochondrial function. The first step in the metabolism of benzoate to hippurate requires ATP, and both steps take place in the mitochondrial matrix. Indeed, Krahenbuhl et al. demonstrated that benzoate metabolism, and thus hippurate formation, was a reflection of hepatic mitochondrial function in rats.[233] Thus, it is possible that impaired mitochondrial functioning may have contributed to the decreased excretion of hippurate and citrate in patients with obstructive jaundice and glomerulonephritis [174, 176]. Additionally, diminished hepatic and renal

mitochondrial function may have been a factor in the reduced excretion of hippurate, citrate, 2-oxoglutarate and succinate documented in investigations of nephrotoxins and hepatotoxins.[201, 204, 210-212]

Table 1: Variation in the excretion of hippurate associated with differences in diet, microbiota, disease states and disorders, toxicity, and parasitic infection. Arrows pointing upwards indicate increased excretion; arrows pointing downwards indicate decreased excretion. H.A; hippuric acid.

Group of interest	Comparison group	H.A excretion	In samples from group of interest relative to controls		Technique(s) used for measuring hippurate	Sample size	Subjects used	Reference
			Other urinary metabolites for which excretion ↑	Other urinary metabolites for which excretion ↓				
Diet								
Black and green tea	Wash out periods	↑	1,3-dihydroxyphenyl-2-O-sulfate, glycine, valine, pyruvate, 2-oxoglutarate, succinate, dimethylamine, *N*-acetyl (glycoproteins)	Glutamine/glutamate	^{1}H NMR spectroscopy	17	Healthy men	97
60% whole-grain wheat flour diet	60% refined wheat flour diet	↑	Tyrosine, tryptophan, creatine, citrate, phenylalanine, fumarate	Pyruvate, taurine	^{1}H NMR spectroscopy	2 groups (n = 10/group)	Male Wistar rats	103
Standard phytochemical diet (SPD)	Low phytochemical diet (LPD)	↑		Creatinine and methyl histidine	^{1}H NMR spectroscopy and HPLC-MS	21 (12 women, 9 men)	Healthy women and men	102
Lacto-vegetarian subjects	Omnivorous subjects	↑	Succinate, citrate, formate	TMAO, taurine, glycine, phenylalanine, methyl histidine	^{1}H NMR spectroscopy	161 (78 women, 83 men)	Healthy men and women	70
Microbiota								
Suppression of microbiota with antibiotics	Saline/vehicle injection	↓	Guanidoacetic acid	m-HPPA	^{1}H NMR spectroscopy	Controls vs antibiotics (n = 10/group)	Male Wistar rats	115
						Controls vs antibiotics (n = 6/group)	Female outbred NMRI strain mice	113
Germ-free animals	Conventional animals	↓	Creatinine	3-hydroxycinnamic acid, PAG, 4-hydroxypropionic acid, *N*-acetylated	^{1}H NMR spectroscopy	Conventional vs germ-free (n = 5/group)	C3H/HeJ mice	111

				glycoprotein				
Germ-free animals introduced to non-sterile environment/inoculated with intestinal microbiota	Germ-free urinary profiles	↑	Trimethylamine N-oxide, Phenylacetylglycine, 3-hydroxypropionic acid, benzoic acid		^{1}H NMR spectroscopy	3	Male Fischer 344 germ-free rats	112
Disease states and disorders:								
Obesity								
Obese individuals	Healthy controls	↓	2-hydroxyisobutyrate	Trigonelline, xanthine	^{1}H NMR spectroscopy	25 (15 obese, 10 controls)	Men	143
Obese Zucker (fa/fa) rats	Lean Zucker (fa/-) and (-/-) rats	↓	α-hydroxy-*n*-butyrate, lactate, fumarate, citrate, free fatty acids, DMA, dimethylglycine, valine, lysine, glutamine/glutamate, succinate/malate, formate, 3-methylglutarate, propionate, acetate	2-oxoglutarate, phenylacetylglycine (PAG), allantoin,N-acetylglycoprotein, proline, ornithines, creatinine, pyridoxine, guanidoacetate	^{1}H NMR spectroscopy	16 (n = 8 for lean and obese Zucker rats)	Male Zucker rats	144
					^{1}H NMR spectroscopy	24 (n = 8 per genotype)	Male Zucker rats	145
Diabetes								
Children with type 1 diabetes	Healthy controls	↑	Citrate, alanine, lactate		^{1}H NMR spectroscopy	50 (n = 25/group)	Children with type 1 diabetes and age- and sex-matched controls	146
Type II diabetic patients	Healthy controls	↑	Lactate, citrate, glycine, alanine, TMAO, dimethylamine, creatine, acetate, betaine, phenylalanine, tyrosine	Glutamate/glutamine, N-methyl nicotinamide, uridine.	^{1}H NMR spectroscopy	53 (33 type II diabetic patients, 20 controls)	Type II diabetic patients	149
					^{1}H NMR spectroscopy	32 (16 patients, 16 healthy subjects)	Type II diabetic patients	148

Blood pressure								
Subjects with high blood pressure	(correlation)	↓	Alanine (positive correlation with blood pressure)	Formate (inverse correlation)	^{1}H NMR spectroscopy	4,630 participants	Men and women in China, Japan, UK and USA.	151
Spontaneously hypertensive rats	Wistar Kyoto controls	↓		Citrate, 2-oxoglutarate	^{1}H NMR spectroscopy	12 (n = 6/group)	Spontaneously hypertensive and Wistar Kyoto rats	152
Gastrointestinal diseases								
Crohns disease (CD) and ulcerative colitis (UC) patients	Healthy controls	↓	Formate	4-cresol sulfate	^{1}H NMR spectroscopy	206 subjects (86 CD patients, 60 UC patients, 60 healthy controls)	Male and female CD and UC patients, and healthy controls	166
Renal function								
Patients with obstructive jaundice	Healthy controls	↓	3-hydroxybutyrate, acetate	Citrate	^{1}H NMR spectroscopy	75 (35 patients, 40 healthy individuals)	Male and female patients and healthy controls	172
Patients with glomerulonephritis	Healthy controls	↓	Lactate, acetate, TMAO	Citrate, glycine, and creatinine		77 patients, 85 controls		174
Patients with primary renal hypouricemia	Healthy controls	↓	Phenylalanine, alanine, glycine, glutamate, acetate	Creatinine, TMAO		36 patients, 39 sex- and age-matched healthy individuals		175
Psychological disorders								
Individuals with high anxiety traits	Individuals with low-anxiety traits	↑	Glycine, citrate, 3-methoxytyrosine, β-alanine, proline, 3,4-dihydroxyphenylalanine (DOPA), adrenaline	Methyl-succinate, trans-aconitate, p-cresol sulfate	^{1}H NMR spectroscopy	30 subjects (13 high anxiety, 17 low anxiety)	Male and females with high or low anxiety traits	182

Chronic unpredictable mild stress-treated rats	Control rats (housed together)	↑	Kynurenic acid, xanthurenic acid, phenylalanine, N_2-succinyl-$_L$-ornithine, phenylacetylglycine	Tryptophan, indoxyl sulfate, indole-3-acetate, citrate, 2-oxoglutarate, creatinine	UPLC-MS	16 (n = 8/group)	Male Sprague–Dawley rats	184
Autism								
Autistic children	Healthy controls	↓	*N*-methyl-2-pyridone-5-carboxamide, *N*-methyl nicotinic acid, and *N*-methyl nicotinamide, taurine	Glutamate, phenyacetylglutamine.	^{1}H NMR spectroscopy	39 autistic children, 28 nonautistic siblings	Male and female children	190
Toxicity								
Nephrotoxins	pre-dose urine sample/vehicle controls	↓	Glucose, glycine, alanine, histidine, lactate, acetate, succinate, TMAO	Citrate, creatinine, 2-oxoglutarate	^{1}H NMR spectroscopy	11	Patients who received ifosfamide chemotherapy	200
					^{1}H NMR spectroscopy	36 (6 treatment groups, n = 6/group)	Male Wistar rats	202
Nephrotoxins (which act as antibiotics)	Vehicle controls	↓	Glucose, lactate, acetoacetate, alanine, valine, lysine,glycine, glutamine/glutamate, citrate	Allantoin	^{1}H NMR spectroscopy	12 (4 groups, n = 3/group)	Male Fischer 344 rats	194
					^{1}H NMR spectroscopy	12 (n = 3/group)	Male New Zealand White rabbits	196
Hepatotoxins	Vehicle controls	↓	Alanine, creatine, taurine	2-oxoglutarate, citrate, succinate, TMAO	^{1}H NMR spectroscopy	10 (n = 5/group)	Male Sprague–Dawley rats	209
					^{1}H NMR spectroscopy	20 (n = 5/group)	Male Han Wistar rats	210
					^{1}H NMR spectroscopy	15 (n = 3/group)	Male Crj:CD (SD) rats	208
Parasitic infection								
S. mansoni, S. japonicum, T. brucei brucei, E. caproni and *N. americanus* infection	uninfected mice	↓	TMA, PAG, pyruvate, p-cresol glucuronide	Citrate, succinate, creatine, taurine, acetate, 2-ketoisocaproate, butyrate	^{1}H NMR spectroscopy	20 (n = 10/group)	Female mice (NMRI strain)	88
						36 (n = 18/group)	Male Syrian SLAC hamsters	224
						24 (n =	Female NMRI	225

						12/group)	mice	
						24 (n = 12/group)	Female out-bred NMRI mice	227
						20 (n = 10/group)	Male Syrian SLAC hamsters	228
						20 (n = 10/group)	Male golden hamsters	229

Co-variation in the excretion of hippurate and TCA cycle intermediates has been seen in other diverse subject areas; with decreased excretion observed in studies of hypertension and parasitic infection[153, 225-226, 231]; and increases observed in studies of diet, diabetes and psychological disorders.[70, 97, 103, 147, 184]

In the study of SHR by Akira et al., it was found that SHR excreted reduced concentrations of hippurate, citrate and 2-oxoglutarate, compared to normotensive Wistar Kyoto rats.[153] The authors speculated that the reduced citrate excretion may have been due to increased proximal tubular reabsorption of this metabolite, or also alteration of the TCA cycle. The reduced excretion of hippurate in the SHR strain was proposed by the authors to be due to strain-related differences in the microbiomes of the animals. Nevertheless, if the TCA cycle was impaired in the SHR, this may have influenced the rate of metabolism of benzoate, and thus hippurate formation.

Differences in the microbiome were also suggested to underlie reduced hippurate excretion in several studies of parasitic infection.[225-226, 231] However, the authors also cited that the reduced excretion of TCA cycle intermediates could be due to an inadequate acetyl-CoA formation,[225] and "perturbation in mitochondrial function".[226] Accordingly, it is possible that reduced mitochondrial ability for glycine conjugation of benzoate also contributed to the reduced excretion of hippurate observed in these studies.

Conclusions

Together with established connections with dietary components, hepatic function and toluene exposure, hippurate has been associated with a diverse range of disease states and alterations in metabolism. In particular, the relationship between hippurate excretion and variation in the microbiota is proving to be a significant area for investigation, particularly in light of evidence relating to the differing microbiomes of obese and lean individuals, and also those with

gastrointestinal diseases. Clearly, given the many factors that have been found to be associated with changes in the urinary excretion of hippurate, its use as a biomarker needs to be performed with care. It is not unusual for these variations to be accompanied by changes in the excretion of other metabolites (Table 1). It is likely that the value of urinary hippurate as a biomarker lies in the conditional relationships between hippurate excretion and the excretion of a variety of other urinary metabolites, with the directional change in concentration of a variety of metabolites in combination, giving rise to a specific co-variation pattern, indicative of the disease state or response to intervention, rather than one metabolite alone. Future developments in analytical techniques and statistical analyses, and the combined use of complementary techniques such as proteomics, metabonomics and metagenomics, will give further insight into the biological significance of urinary metabolites such as hippurate, host endogenous metabolism and intestinal microbial metabolism, in health and disease.

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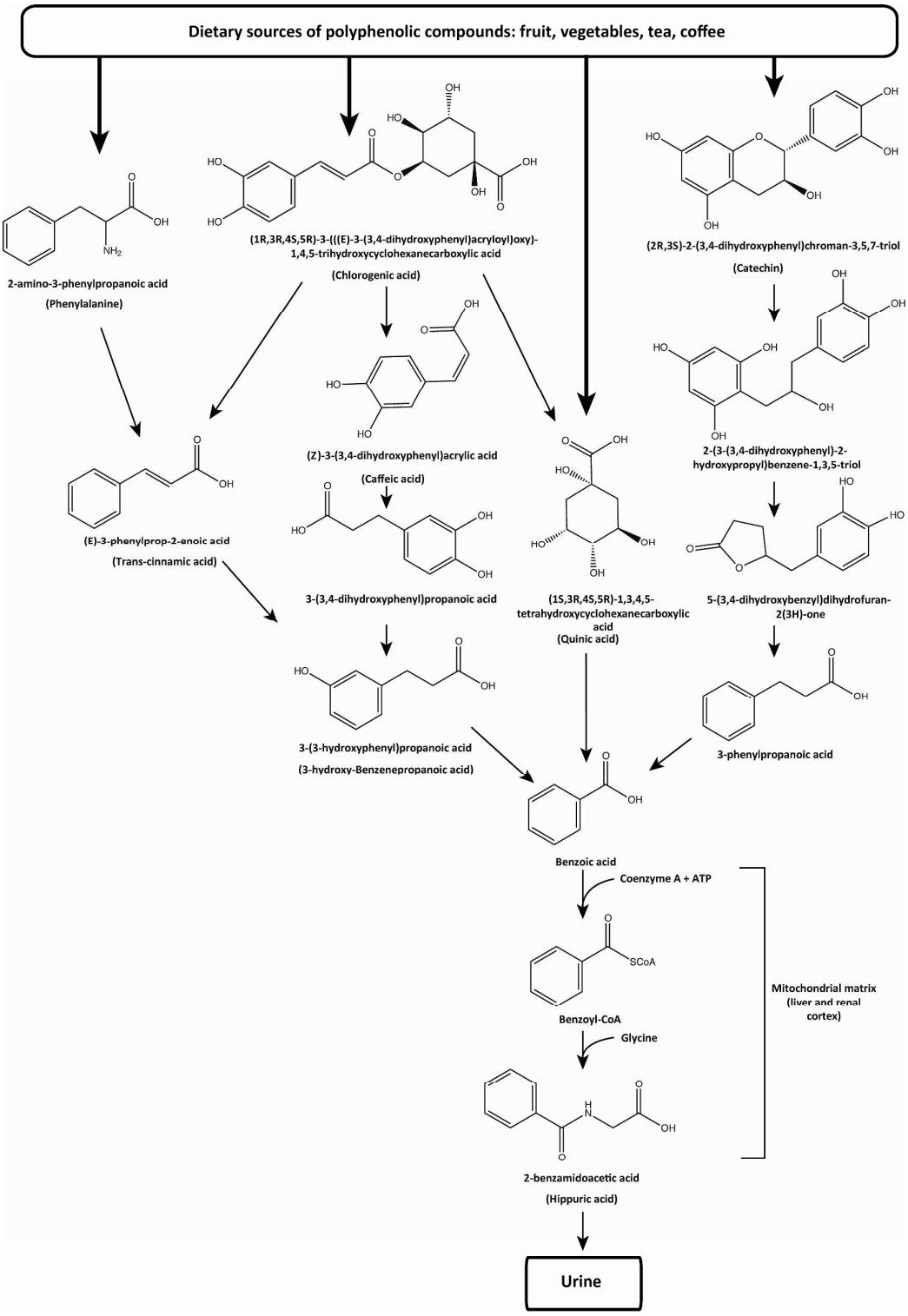
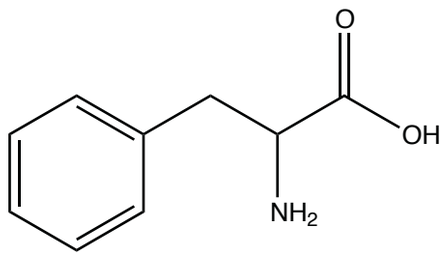
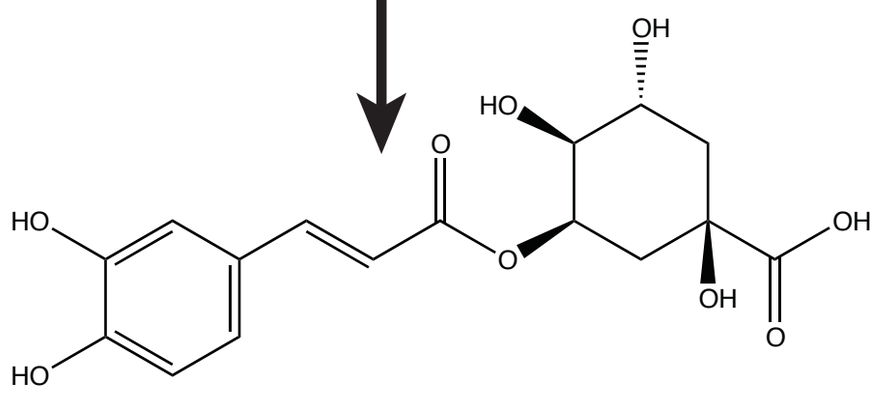

Dietary sources of polyphenolic compounds: fruit, vegetables, tea, coffee
2-amino-3-phenylpropanoic acid
(Phenylalanine)
(1R,3R,4S,5R)-3-(((E)-3-(3,4-dihydroxyphenyl)acryloyl)oxy)-1,4,5-trihydroxycyclohexanecarboxylic acid
(Chlorogenic acid)
(2R,3S)-2-(3,4-dihydroxyphenyl)chroman-3,5,7-triol
(Catechin)
(E)-3-phenylprop-2-enoic acid
(Trans-cinnamic acid)
(Z)-3-(3,4-dihydroxyphenyl)acrylic acid
(Caffeic acid)
2-(3-(3,4-dihydroxyphenyl)-2-hydroxypropyl)benzene-1,3,5-triol
3-(3,4-dihydroxyphenyl)propanoic acid
(1S,3R,4S,5R)-1,3,4,5-tetrahydroxycyclohexanecarboxylic acid
(Quinic acid)
5-(3,4-dihydroxybenzyl)dihydrofuran-2(3H)-one
3-(3-hydroxyphenyl)propanoic acid
(3-hydroxy-Benzenepropanoic acid)
3-phenylpropanoic acid
Benzoic acid
Coenzyme A + ATP
Benzoyl-CoA
Glycine
Mitochondrial matrix (liver and renal cortex)
2-benzamidoacetic acid
(Hippuric acid)
Urine

Dietary sources of polyphenolic compounds: fruit, vegetables, tea, coffee



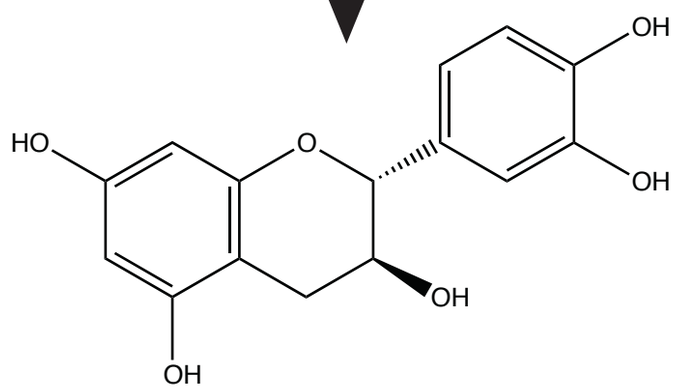
2-amino-3-phenylpropanoic acid

(Phenylalanine)



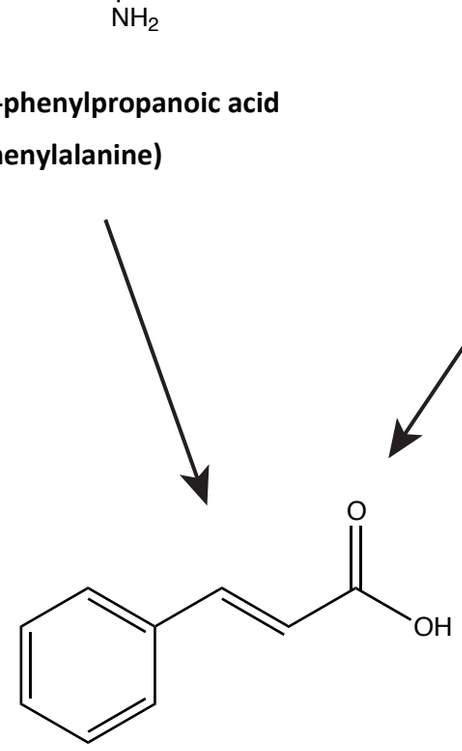
(1R,3R,4S,5R)-3-(((E)-3-(3,4-dihydroxyphenyl)acryloyl)oxy)-1,4,5-trihydroxycyclohexanecarboxylic acid

(Chlorogenic acid)



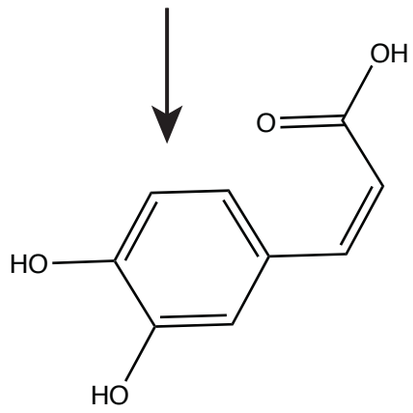
(2R,3S)-2-(3,4-dihydroxyphenyl)chroman-3,5,7-triol

(Catechin)



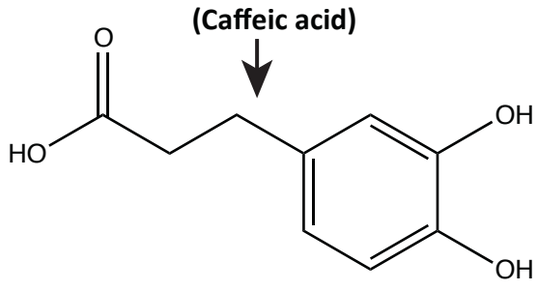
(E)-3-phenylprop-2-enoic acid

(Trans-cinnamic acid)

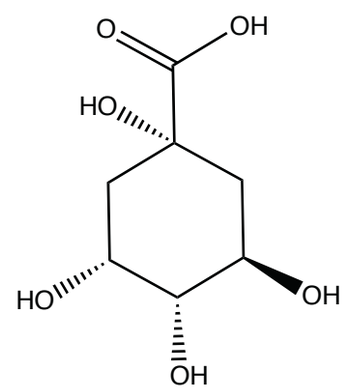


(Z)-3-(3,4-dihydroxyphenyl)acrylic acid

(Caffeic acid)

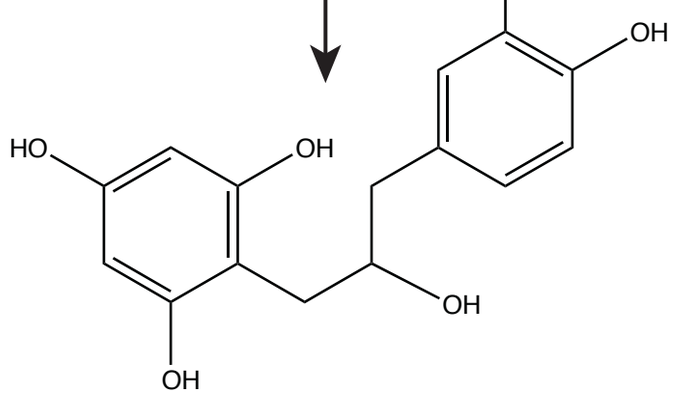


3-(3,4-dihydroxyphenyl)propanoic acid

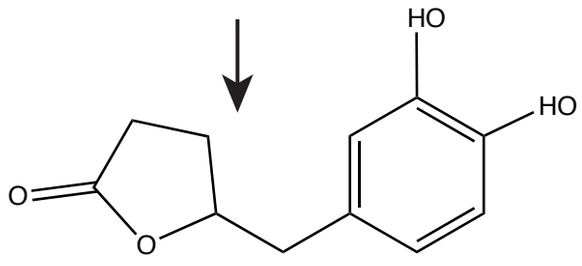


(1S,3R,4S,5R)-1,3,4,5-tetrahydroxycyclohexanecarboxylic acid

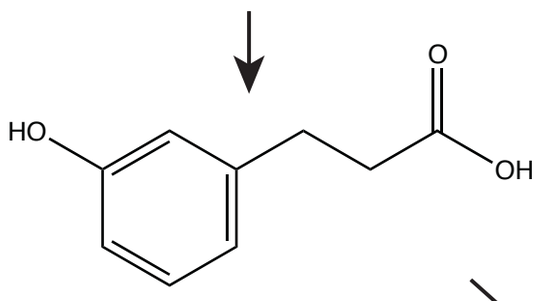
(Quinic acid)



2-(3-(3,4-dihydroxyphenyl)-2-hydroxypropyl)benzene-1,3,5-triol

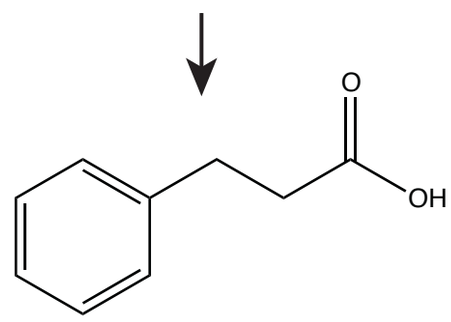


5-(3,4-dihydroxybenzyl)dihydrofuran-2(3H)-one

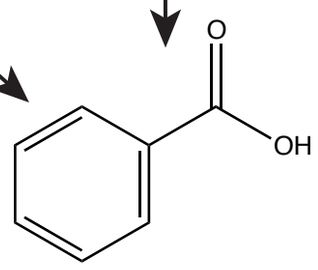


3-(3-hydroxyphenyl)propanoic acid

(3-hydroxy-Benzenepropanoic acid)

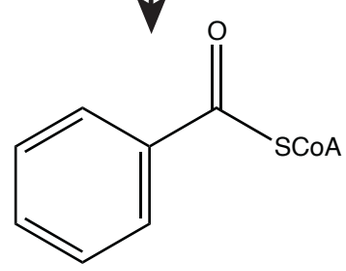


3-phenylpropanoic acid



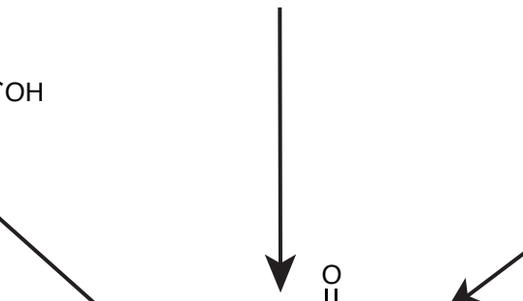
Benzoic acid

Coenzyme A + ATP



Benzoyl-CoA

Glycine

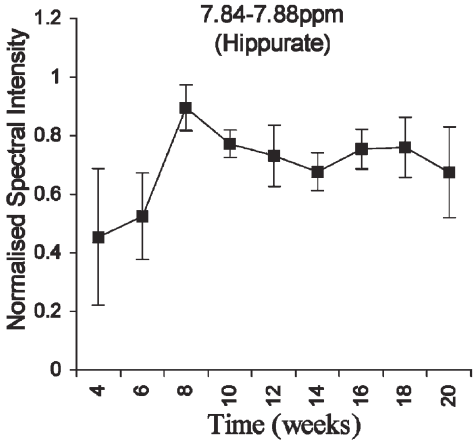


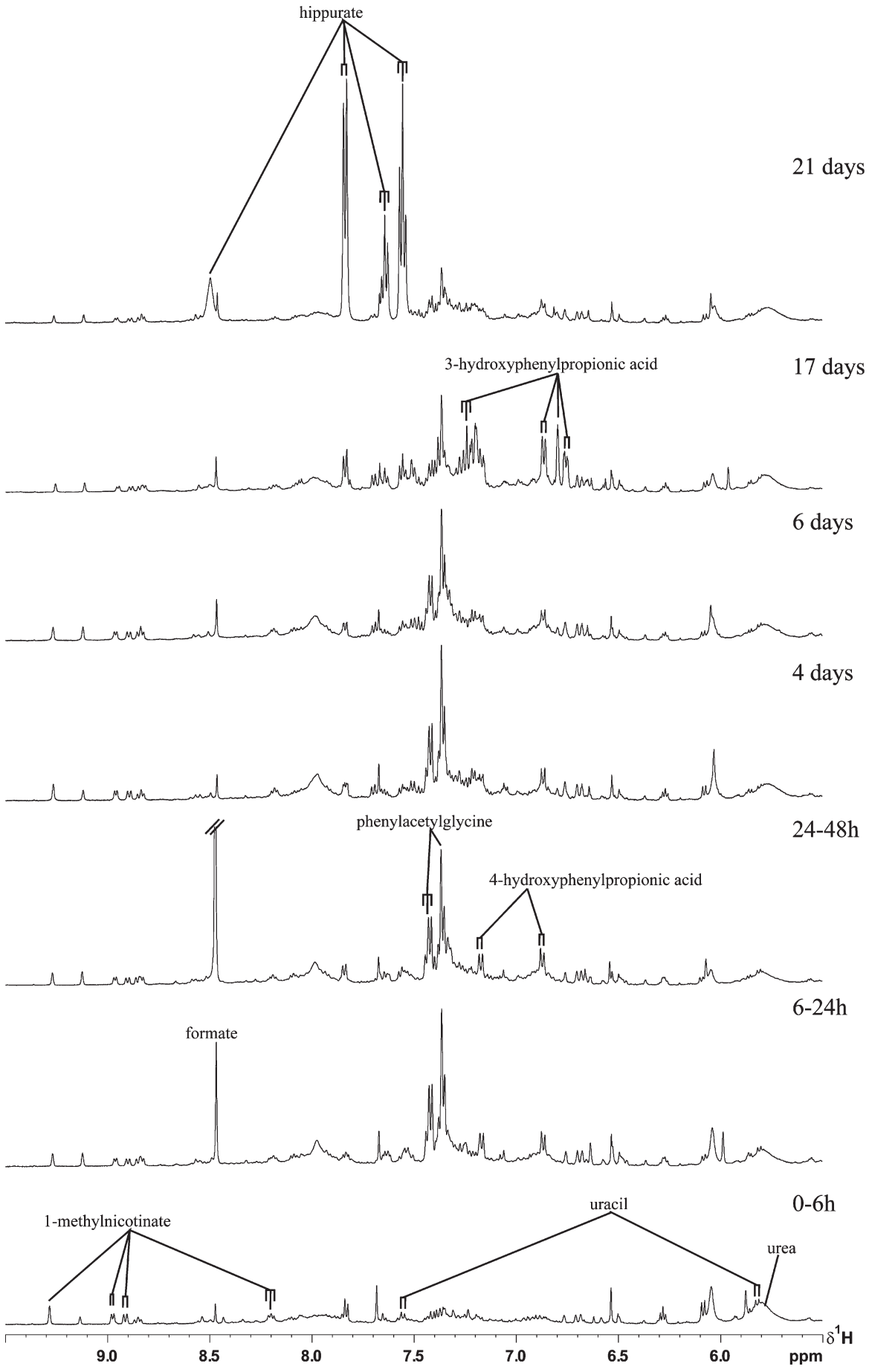
2-benzamidoacetic acid

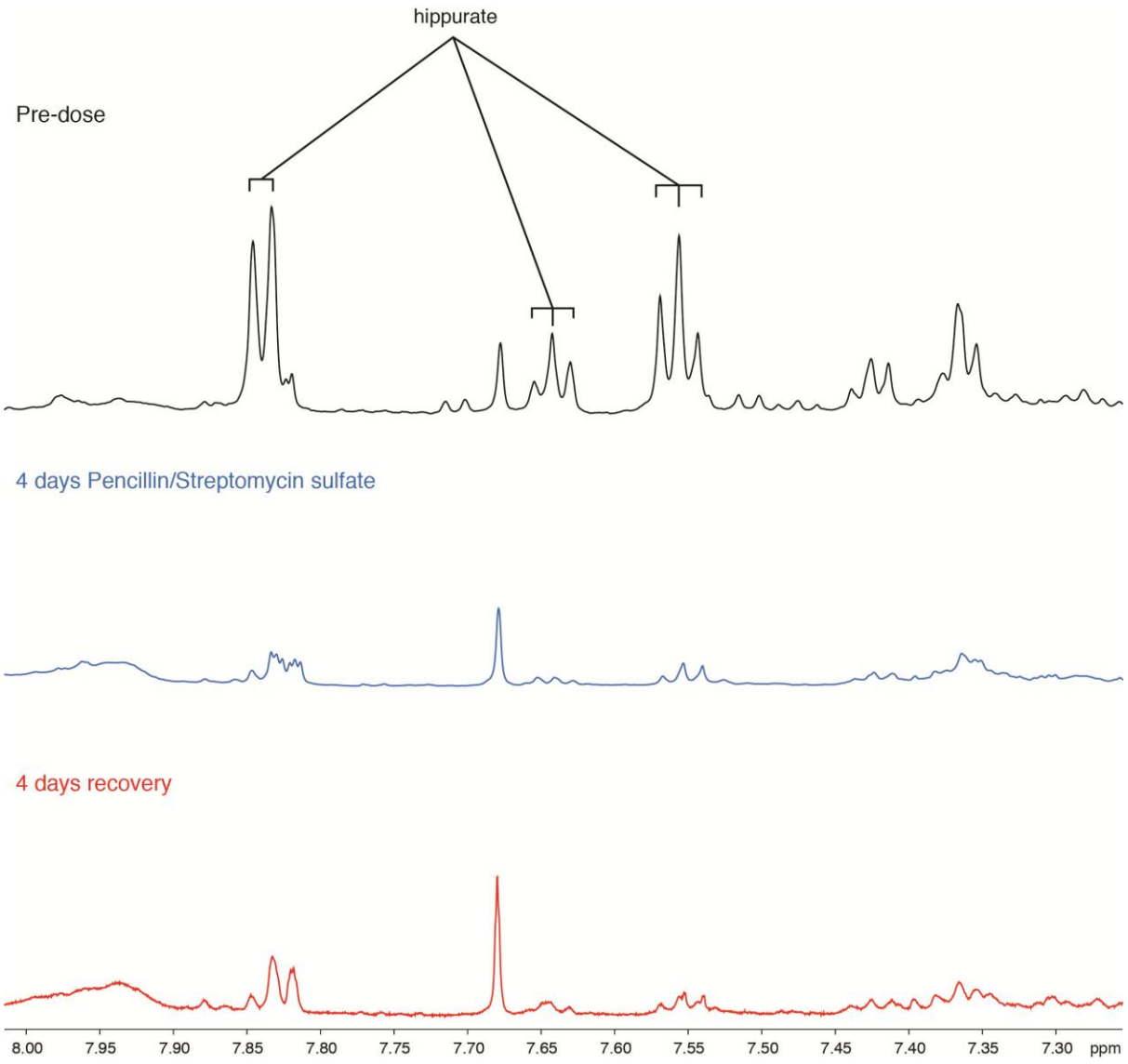
(Hippuric acid)

Urine

Mitochondrial matrix (liver and renal cortex)


7.84-7.88ppm
(Hippurate)
Normalised Spectral Intensity
1.2
1
0.8
0.6
0.4
0.2
0
4
6
8
10
12
14
16
18
20
Time (weeks)


hippurate
21 days
3-hydroxyphenylpropionic acid
17 days
6 days
4 days
phenylacetylglycine
4-hydroxyphenylpropionic acid
24-48h
6-24h
formate
0-6h
uracil
1-methylnicotinate
urea
δ¹H
9.0
8.5
8.0
7.5
7.0
6.5
6.0
ppm


hippurate
Pre-dose
4 days Pencillin/Streptomycin sulfate
4 days recovery
8.00
7.95
7.90
7.85
7.80
7.75
7.70
7.65
7.60
7.55
7.50
7.45
7.40
7.35
7.30
ppm